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Emergence of a multi host biofilm forming opportunistic pathogen *Staphylococcus sciuri* D26 in coral *Favites abdita*

S. Divya[a], T. Thinesh[a,b], G. Seghal Kiran[c], Saqib Hassan[a], Joseph Selvin[a,*]

[a] Department of Microbiology, School of Life Sciences, Pondicherry University, Puducherry, India
[b] Florida International University, Miami, FL 33199, USA
[c] Department of Food Science and Technology, School of Life Sciences, Pondicherry University, Puducherry, India

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ABSTRACT

Corals are hotspots of ocean microbial diversity and imbalance in the composition of coral associated microbes has been mostly correlated with the emergence of climate change driven diseases which affect the overall stability of the reef ecosystem. Coral sampling was performed by SCUBA diving at Palk Bay (latitude 9.271580, longitude 79.132203) south Indian coast. Among the 54 bacterial isolates, an isolate MGL-D26 showed comparatively high biofilm formation and was identified as *Staphylococcus sciuri* based on phylogenetic analysis. The production of exopolysaccharide (EPS) confirmed the formation of a slimy EPS matrix associated with the biofilm. The biofilm formation in *S. sciuri* D26 was induced significantly by UV exposure followed by other stress factors including pollution, agitation, and salinity. The strain inhibited innate immune factors of corals such as melanin synthesis and phenoloxidase. Challenge experiments in a model organism *Aiptasia* sp. showed pathogenicity of *S. sciuri*. Histopathological analysis revealed tissue invasion by *S. sciuri* which was a predisposing factor leading to mortality in challenged *Aiptasia* sp. However, specific disease condition of corals infected by *S. sciuri* requires continuous field monitoring and further investigation. Based on the findings, *S. sciuri* was a first reported multi-host opportunistic pathogen which has emerged in corals under environmental stress.

1. Introduction

Coral reefs are one of the most diverse ecosystems of the earth which harbor a diverse number of microorganisms most of which have remained unexplored [1]. The importance of associations between microorganisms and their invertebrate hosts is becoming increasingly apparent. Although interactions between microorganisms and vertebrates have been well studied, relatively little attention has been given to the examination of microbial–invertebrate association. Recently, researchers have revealed that corals harbor diverse microorganisms which may play a crucial role in coral health. Particularly, bacteria associated with the corals are considered as indicators for the environmental changes [2]. Coral associated microbes have been found to be involved in carbon and nitrogen fixation, metabolism of dimethylsulfoniopropionate and dimethyl sulphide and in coral defense against various pathogens [3]. Even though these coral associated microbes are beneficial to corals, recent findings have suggested that these beneficial microbes could turn into opportunistic pathogens and can cause severe diseases in corals under unfavourable conditions such as elevated temperature, UV radiation, salinity, pH, pollution etc. [4].

Scleractinian coral *Favites* sp. belonging to the family Merulinidae mostly found in Indo-Pacific region and the Indian Ocean [5]. The members of *Favites* sp. are considered to be resilient and are less prone to numerous threats that most of the corals face today. Black band disease, a lethal tissue degrading disease has been reported in *Favites* sp. But environmental changes like climatic conditions and ocean acidification suggest re-considering the resilience of the species to the other threats [6]. Very few attempts have been made to detect the pathogenesis of opportunistic pathogens associated with corals [7]. Climate change plays a major role in causing stress to corals resulting in the emergence of opportunistic pathogens and ultimately coral death. Some infections are particularly daunting for corals such as elevated temperature environments [8–10]. In this background, this study was carried out to elucidate the presence of opportunistic pathogens in healthy corals and evaluate their probable role in pathogenesis and causing coral diseases under adverse conditions. An opportunistic coral pathogen *S. sciuri* was found associated with healthy corals. Albeit report on *S. sciuri* causing coral disease is not available in the literature, this study revealed its virulence cascade and pathogenesis in model organism *Aiptasia* sp. which ultimately supports the hypothesis that *S.*

* Corresponding author.
E-mail addresses: josephselvinss@gmail.com, jselvin.mib@pondiuni.edu.in (J. Selvin).

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sciuri associated with healthy corals can act as an opportunistic pathogen causing disease in corals during the unhealthy environmental conditions around the corals.

2. Materials and methods

2.1. Field monitoring and sample collection

The study site Palk Bay is located near Mandapam, Gulf of Mannar, Marine National Park (latitude 9.271580, longitude 79.132203) in the southeast coast of India (Refer supplementary Fig. 1). This site was monitored for two months (April 2016 and May 2016) during El Nino southern oscillations for the emergence of opportunistic pathogens in healthy corals. A Coral sample measuring 0.5–1 cm^3 of *Favites abdita* was collected from the sampling site Palk Bay. The samples were suspended in sterile phosphate buffered saline and immediately brought to the Microbial Genomics laboratory, Pondicherry University for bacterial isolation.

2.2. Isolation of coral associated bacteria

For isolation of coral associated bacteria coral extract was prepared using sterile seawater and was serially diluted in sterile aged seawater and 100 μl of 10^8 dilutions was plated on Nutrient agar (Himedia) supplemented with 2% NaCl, Zobell Marine agar (Himedia) and Luria Bertani agar (Himedia) plates (pH 7.8) using spread plate technique and were incubated at 37 °C for 24 h. Pure cultures of bacterial colonies were isolated from the sub-cultured plates and colonies with distinct morphologies were stored in glycerol stock and in nutrient agar slants [11,12].

2.3. In vitro assays to evaluate virulence factors in coral associated bacteria

2.3.1. Cell surface hydrophobicity

Cell surface hydrophobicity was determined by standard protocols; the cell pellets obtained from overnight bacterial culture were suspended in phosphate buffered saline (PBS pH 7.2). The cell suspension was mixed with xylene (1:1) and vortexed for 30 s. After 30 min of incubation at 37 °C, the absorbance was measured at 600 nm (Agilent UV–Vis spectrophotometer) [13]. The percentage of cells that bound to xylene was calculated using the following formula,

$$CSH\ (\%) = [1 - (A1/A0)] \times 100.$$

2.3.2. Biofilm formation

Congo red agar method was used for the detection of biofilm formation in coral associated bacteria. The isolates were streaked on the Muller Hinton agar (Himedia) and Brain heart infusion agar supplemented with 0.8 g/l of Congo red dye and incubated for 48 h at 37 °C. The confirmation test on biofilm formation was performed in which bacterial culture was inoculated in 5 ml trypticase soy broth with 1% glucose and the tubes were incubated overnight at 37 °C. After incubation, the tubes were allowed to air dry and were stained with (0.1%) crystal violet. Visible biofilm lined on the wall and the bottom of the test tubes was considered as positive [14]. The positive isolates were quantified using a microtitre plate method [15]. Microscopic observation of bacterial biofilm was done as follows, 10 μl of overnight grown culture in LB broth was dropped carefully on LB agar plate and monitored every 6 h under the stereo microscope (Olympus) for the appearance of wrinkled colony development indicating biofilm formation [16]. Following that biofilm images were observed in scanning electron microscope (SEM) and confocal laser scanning microscope (CLSM). Briefly, 0.5 ml of overnight grown culture was inoculated into 50 ml of LB containing 0.2 gm of glass wool and was allowed to develop biofilm at 37 °C with continuous shaking. After 24 h of incubation, the glass wool was removed from the broth and washed with PBS. The biofilm images were recorded in CLSM. High resolution images were acquired for scanning area of 12 × 12 at a resolution of 1000 × 1000 pixels [17]. The samples for SEM analysis were air dried and fixed in (2.5%) glutaraldehyde with 0.1 M phosphate buffer and 0.1 M cacodylate buffer followed by dehydration using ethanol for 30 min. Then the samples were coated with gold for observation in SEM [18].

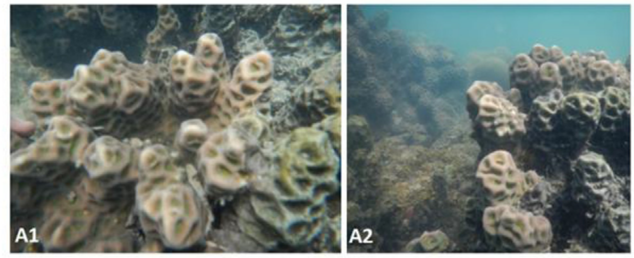

Fig. 1. The *Favites* sp. colony from the site of collection (Palk Bay). The colony found partially diseased and the other portion remains healthy. Fragments from the healthy portion were sampled for the isolation of biofilm forming opportunistic pathogen. A1). Healthy portion of the colony A2). Diseased portion of the colony.

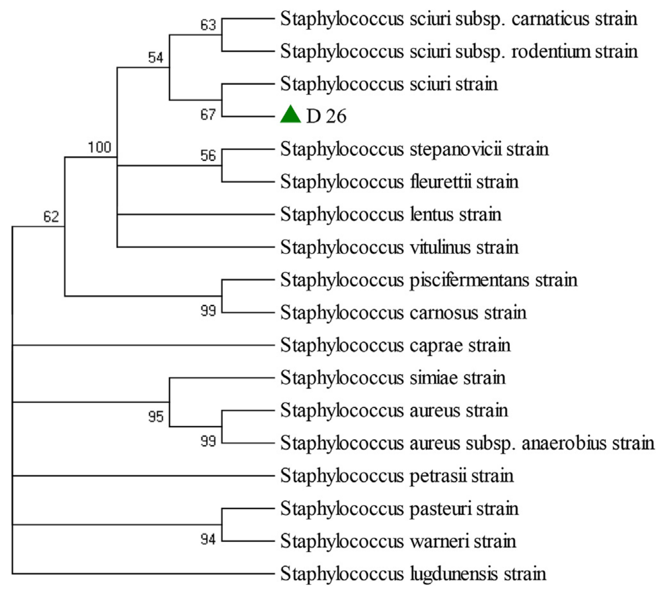

Fig. 2. Phylogenetic tree construction using the Maximum Likelihood method.

2.4. Effect of various stress parameters on biofilm formation by the strain D26

Crystal violet assay was performed to find the influence of various stress factors such as salinity, UV light, temperature, agitation and pollution on biofilm formation. The isolate D26 was inoculated into 5 ml LB broth containing 0.1 gm of glass wool. Then the test tubes were incubated with different stress conditions such as salinity (4%), UV light exposure for 1 h, temperature (4 °C and 65 °C), agitation (1200 rpm) and heavy metal (Zinc, 60 mg/ml) [19]. The stress factors and their ranges were selected based on the preliminary experiments.

2.5. Auto-aggregation and co-aggregation in coral associated bacteria

Auto-aggregation and co-aggregation properties of biofilm forming strain D26 was assessed by standard protocol [20–22]. In brief, cell pellets of overnight grown culture were suspended in PBS and was allowed to stand for 24 h. Absorbance at 280 nm was measured at

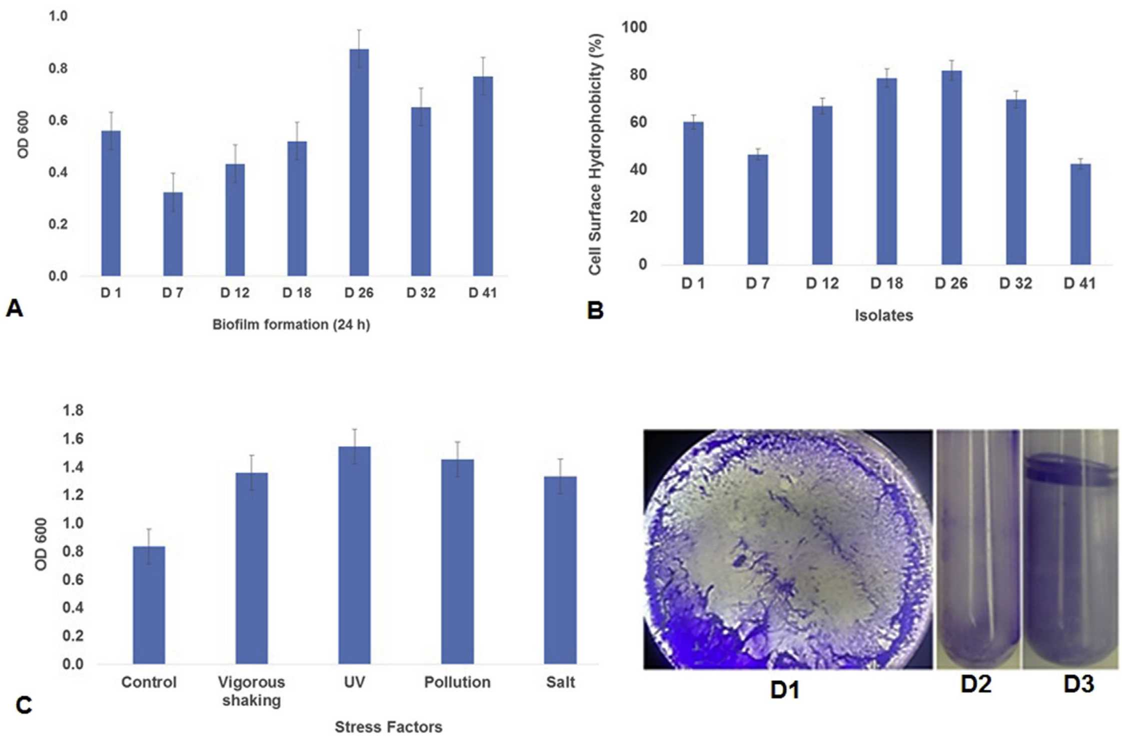

Fig. 3. Biofilm formation potential of the strain D26. A. Among the seven isolates D 26 showed maximum biofilm formation. B. D26 exhibited a maximum percentage of cell surface hydrophobicity. C. Different stress parameters such as vigorous shaking, UV light, Zinc, NaCl was given for the assessment of biofilm forming potential of D26 during stress condition where UV light triggers maximum biofilm formation. D1. Biofilm formation in microtitre plate viewed under light microscope. D2. Tube assay in which the control tube showing no biofilm formation. D3. Tube assay in which the positive tube showing biofilm formation in both air liquid interface and at the bottom.

different time intervals such as 6, 12, 24, and 48 h. For the co-aggregation assay, in addition to the above steps overnight culture of a pathogen *Staphylococcus aureus* MTCC96 was added and the OD was measured in a multimode well plate reader at 280 nm (Versamax Model 2020). The absorbance was recorded at varying intervals of 6, 12, 24 and 48 h. The percentage of aggregation was calculated using the formula:

Aggregation (%) = [1 – (A600 of upper suspension at time t/A600 of total bacterial suspension at time 0)] × 100

2.6. Chemical composition analysis of exo-polysaccharide (EPS)

The EPS production was screened in ATCC 14 media for the formation of slimy mucoid colonies. Characterization of EPS was performed as described [23]; briefly, the cell free supernatant was collected by centrifugation at 10500 × g for 30 min. To the culture filtrate, twice the volume of ice cold ethanol was added and incubated overnight at 4 °C. The resulting precipitate was dissolved in sterile distilled water and dialysed against water. The total carbohydrate content of EPS was estimated using the phenol-sulphuric method and total protein was determined by Bradford method [24]. The EPS extracted was lyophilised (Yamato) and used for FTIR analysis [25].

2.7. Assessment of extra-cellular virulence factors

Overnight grown cultures of the strain D26 were screened for the production of extra-cellular virulence factors such as protease, lecithinase, fibrinolysin, and hemolysin. The superoxide dismutase activity was estimated based on the reduction of nitroblue tetrazolium (NBT) by riboflavin and tetramethylethylenediamine (TEMED) [26].

2.8. Taxonomic identification of the isolate D26

Biochemical characteristics of the isolate D26 were performed based on Bergey's manual identification of bacteria. Grams staining was performed using Gram staining kit (Himedia laboratories) and observed under a light microscope [27]. Motility of bacteria was observed using hanging-drop wet method and Cragie's technique [28] other biochemical characterizations included catalase test, oxidase test, urease test, mannitol salt agar test, fermentation of carbohydrates, resistance of novobiocin and hemolytic activity was performed [29].

PCR amplification of 16S rRNA gene was performed using universal primers 27 F: AGA GTT TGA TCM TGG CTC AG and 1492R: CGG TTA CCT TGT TAC GAC TT. The reaction mix in 50 µl PCR tubes consisting of 5 µl of 10X PCR buffer, 5 µl of 10 mM total dNTPs mixture, 5 µl of 25 mM $MgCl_2$, 2.5 µl of 1 mM of each primer, 5 µl of 1 mg template DNA and 2.5 U of exTaq DNA polymerase and 23 µl of sterile milliQ water. The standard PCR amplification conditions used included an initial denaturation at 95 °C for 5 min; 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), and extension (2 min at 72 °C); and a final extension at 72 °C for 7 min [26]. The sequence of the PCR amplicon was aligned and edited using Bioedit software. The phylogenetic tree was constructed using maximum parsimony algorithm in MEGA version 7.0 (30).

2.9. Ex situ coral experiments to evaluate pathogenic effect of S. sciuri in Favites sp.

Overnight grown culture of D26 (10^8 CFU/ml) was inoculated into the 100 ml flasks containing 50 ml of sterile seawater and coral fragments of *Favites* sp. measuring approximately 2 cm^2. Control was maintained without inoculation of D26. After 24 h of shaking incubation at 37 °C, both coral fragments were washed with ethanol followed by double washing with sterile seawater. The extracts were used for

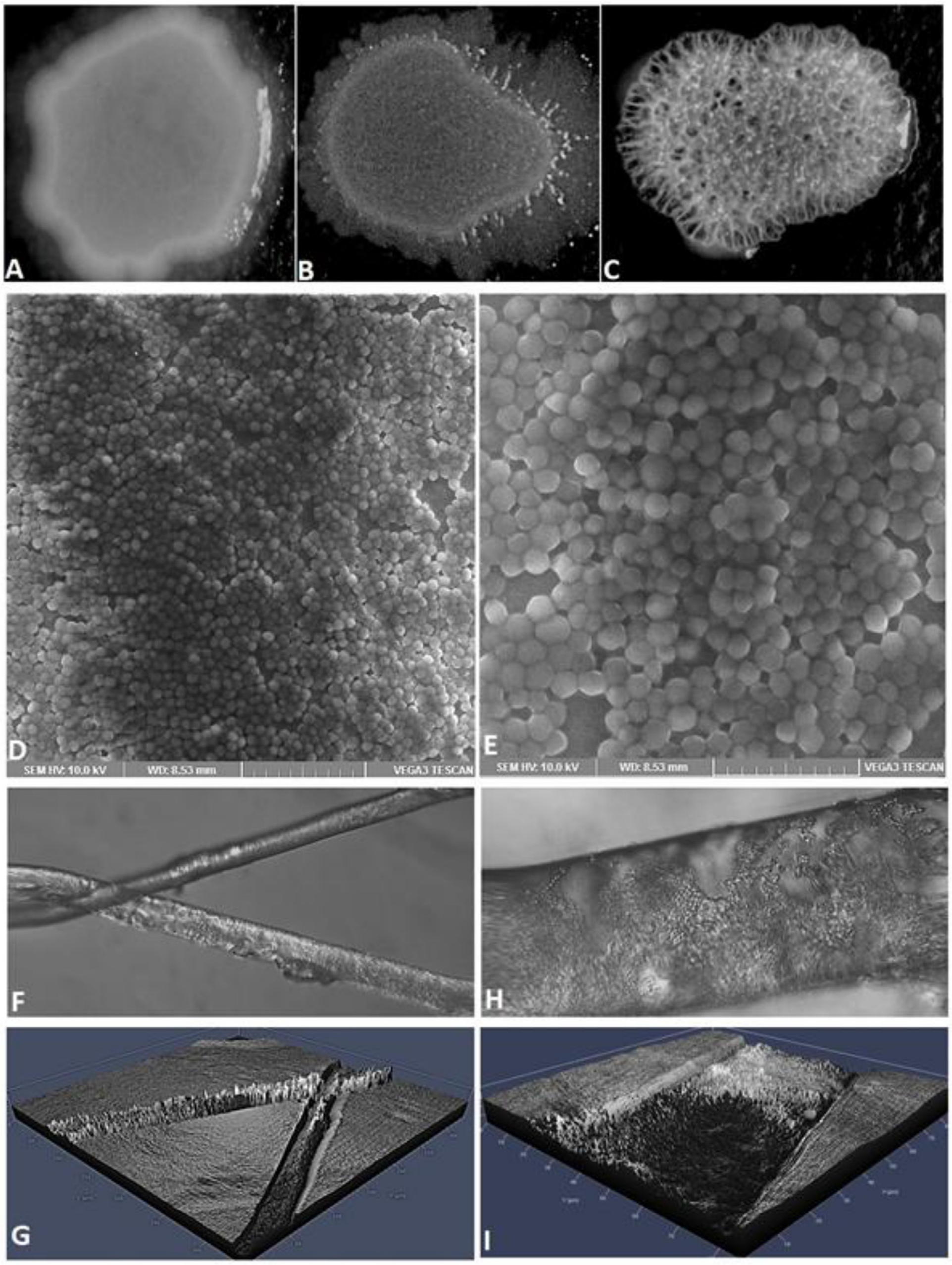

Fig. 4. Biofilm and colony morphology of the strain D26. The panels A–C are showing wrinkled colony development of D26 viewed under stereo microscope. A, B, and C were D26 colony at 12, 24 and 48 h respectively. D and E are showing biofilm formation in glass coverslip viewed under SEM at different magnifications 10 μm and 5 μm respectively. F and H are CLSM view of biofilm formation on glass wool at different magnifications (10 μm and 5 μm). G and I show the tridimensional view under CLSM.

viable count assessment using spread plate technique [31].

The coral tissue was extracted using water-pik method [32] briefly, buffer containing 50 mM PBS (pH 7.8) with 0.05 mM dithiothreitol was added vigorously on coral fragments to collect the coral tissue and then homogenised on ice. The homogenised tissue was centrifuged at 4 °C at 6000 rpm to remove the supernatant from the cellular debris and stored at −80 °C till further analysis.

Inhibition of phenoloxidase was assessed as per the modified protocol [33]. Briefly, 200 μl of the coral extract was diluted with 300 μl of 50 mM PBS. After incubation for 15 min at 28 °C, 300 μl of L-tyrosine was added and resulting absorbance was measured immediately at 410 nm. The % inhibition was calculated by using the formula.

Percentage inhibition of phenoloxidase (%) = (Control- Test)/Control ×100

The coral tissue extract was treated with 0.5 ml of 10 M NaOH and the mixture was vortexed frequently with regular intervals and incubated for 24 h at 37 °C. The mixture was dried at 50 °C. The dried extract was dissolved in 1 ml of deionised sterile water and resulting absorbance was measured at 490 nm. A standard curve was constructed using a melanin standard for calculating the amount (μg) of melanin produced in both control and test samples.

2.10. Challenge experiments in a model organism Aiptasia sp.

The *Aiptasia* polyps were transferred into two petriplates containing sterile seawater and were acclimatized for 24 h at 25 °C. The

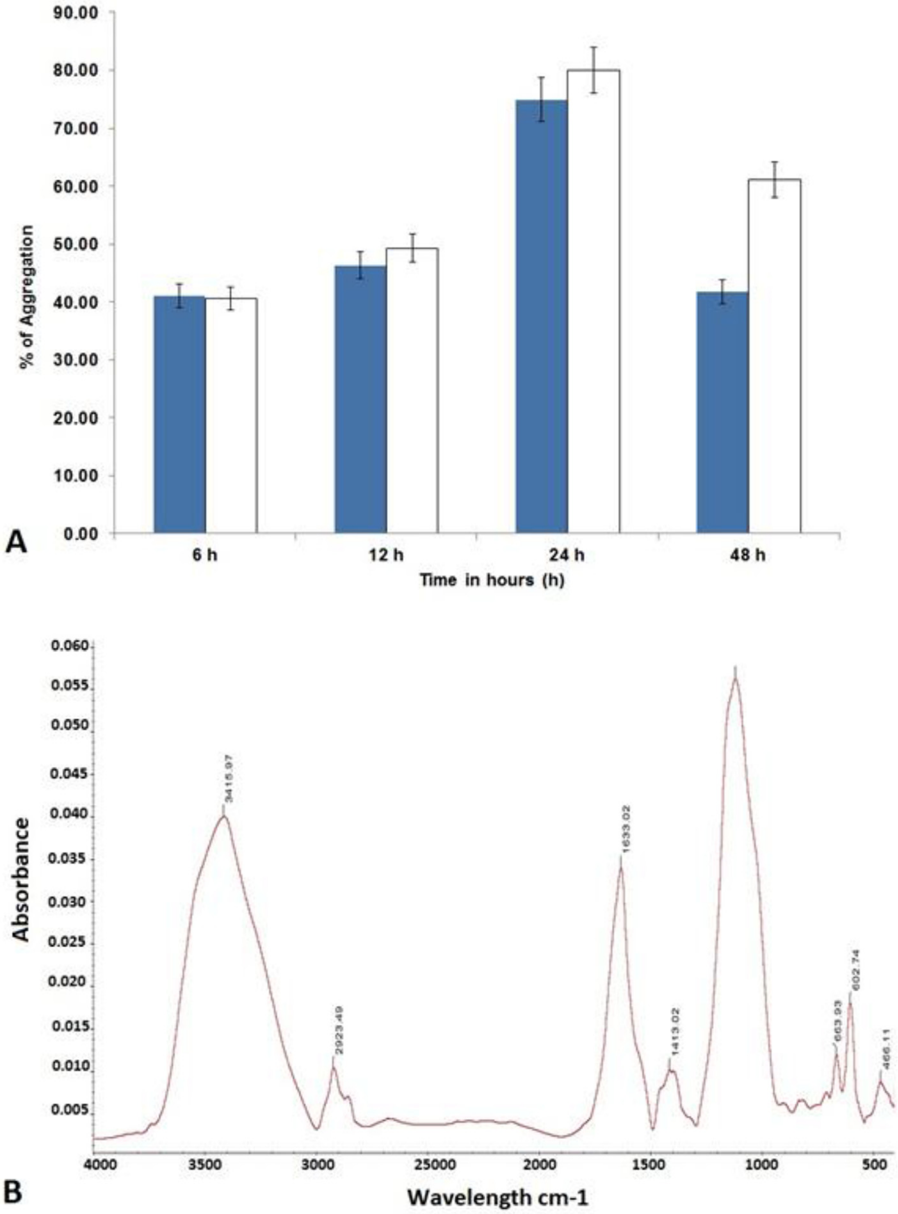

Fig. 5. A) Auto-aggregation potential of biofilm forming isolate D26 and co-aggregation with other biofilm forming isolate D25. B) Fourier transform infrared spectroscopy (FTIR) spectra of water soluble extracellular polymeric substances extracted from biofilm forming isolate D26.

acclimatized *Aiptasia* were transferred into 1 l glass aquaria and inoculated overnight culture of *S. sciuri* (10^8 CFU/ml). A control aquarium was maintained with *Aiptasia* in sterile seawater without challenge. All experiments were conducted in triplicates. Both infected and control tissue samples were processed for histopathological observation. Tissue processing and Hematoxylin and Eosin (H&E) staining was performed as per the standard protocols. The slides were then examined under light microscope to compare control and test specimens [34].

2.11. Statistical analysis

One-way ANOVA was performed for evaluating the comparison between various parameters. P values of < 0.05 were considered to be statistically significant.

3. Result and discussion

3.1. Isolation of coral associated bacteria from Favites sp.

Favites abdita is a stony coral known as larger star coral. This coral is a native species of Indo-Pacific Ocean; its geographical distribution is extended to the Red Sea and East Africa. This species is found to be more resilient than other coral species in Palk Bay. There is no precise information on population trends but this coral comes under 'near-threatened' category as per International Union for Conservation of Nature (IUCN) records [35,36]. Most obvious factors threatening *Favites* include global warming, ocean acidification, and degradation of its coral reef habitats [37]. In this study, the sampling of coral *Favites* sp. was performed during El Nino southern oscillations in Palk Bay to reveal the emergence of opportunistic pathogens from the coral associated bacteria. El Nino events cause bleaching in corals leading to mortality due to increased temperature and direct solar insolation up to 31 °C. Fig. 1 shows the coral collected from Palk bay during May and

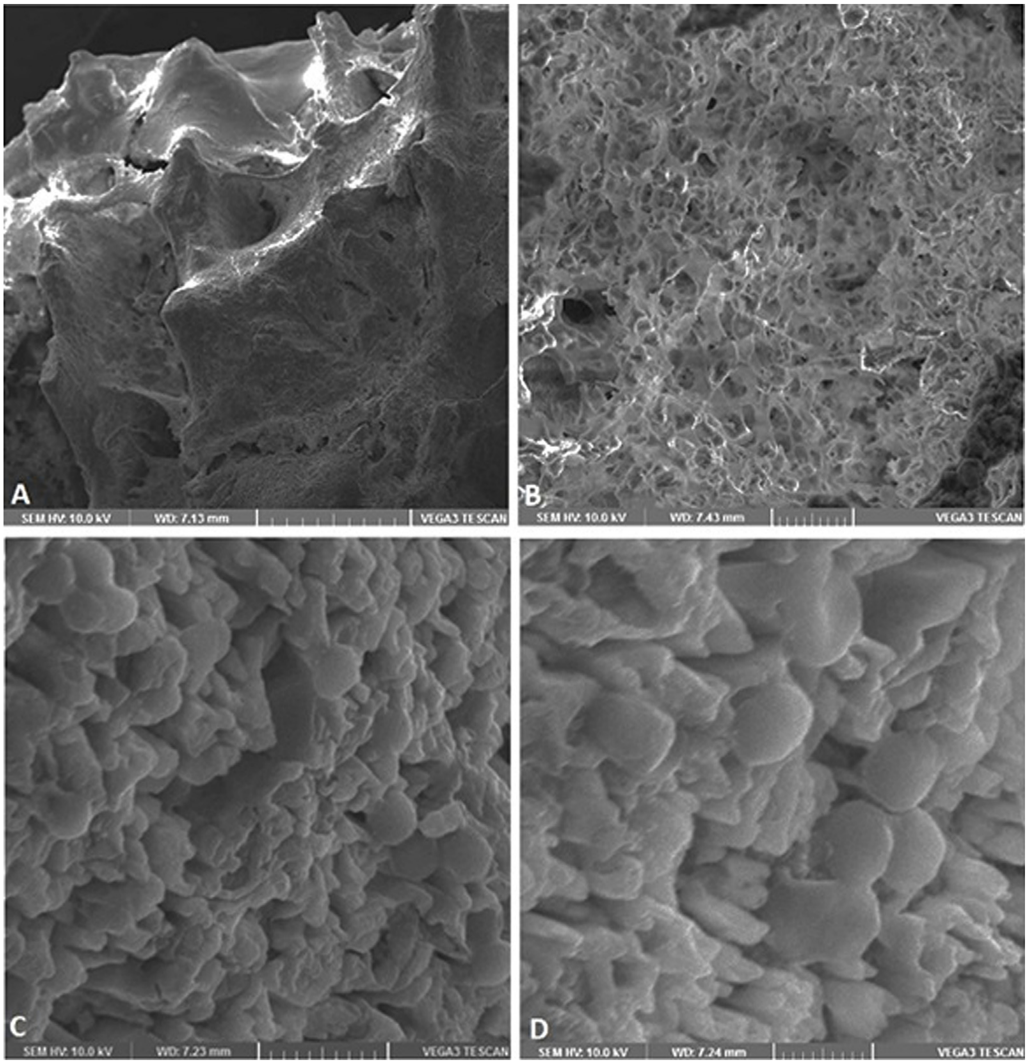

Fig. 6. Coral-bacterial cell adherence. SEM images of coral fragments measuring approximately 1 × 1 cm were treated with *S. sciuri* for 48 h. Different magnifications showed adherence on the surface of the coral, loss of tissues and intrusion of *Staphylococcus sciuri* into the skeleton. A. Surface of the treated coral fragment. B. Porous tissue after treatment due to infection. C, D. Adherence of *S. sciuri* on and into the coral skeleton.

June 2016. The colony found partially diseased (A2) and the other portion remains healthy (A1). Fragments from the healthy portion were sampled for the isolation of biofilm forming opportunistic pathogen. A total of 54 bacterial isolates with distinct morphologies were isolated from *Favites* sp. using serial dilution and spread plate technique. The bacterial colonies were maintained as a pure culture in the nutrient agar slants at pH 7.8 for further assessment.

The overall health status of coral holobiont depends on the interactions between native microorganisms and opportunistic pathogens [38]. Microbial diseases are one of the important causes of coral decline worldwide. In accordance with the present study, Frydenbrog et al. [39] have also reported that elevated temperature may be favouring pathogenic bacteria dominance and may lead to disease in corals. Many species of bacteria have been found to cause specific coral diseases. Among them, most of them are opportunistic pathogens causing infection to the corals in stress condition [40,41].

3.2. Identification of the coral pathogen D26

The biochemical characterization revealed that the bacterial isolate D 26 was a coagulase-negative, *Staphylococcus* sp. (Fig. 2a). The sequence obtained from 16srRNA sequencing was used to construct the phylogenetic tree (Fig. 2) and was deposited in the GenBank with the accession number KX225390. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.6533)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 77.9898% sites). The analysis involved 18 nucleotide sequences. Codon positions included were 1st +2nd+3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 873 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [30].

3.3. Analysis of S. sciuri D26 biofilm

Bacterial biofilm formation is a process in which bacteria colonize on surfaces, get irreversibly attached to the surfaces and produce a slimy EPS matrix where the biofilm cells reside in [23]. Biofilm formation is a pathogenic factor relied on environmental conditions [42]. In this study, *S. sciuri* D26 was characterized as a biofilm forming opportunistic coral pathogen. Biofilm forming potential of D26 was

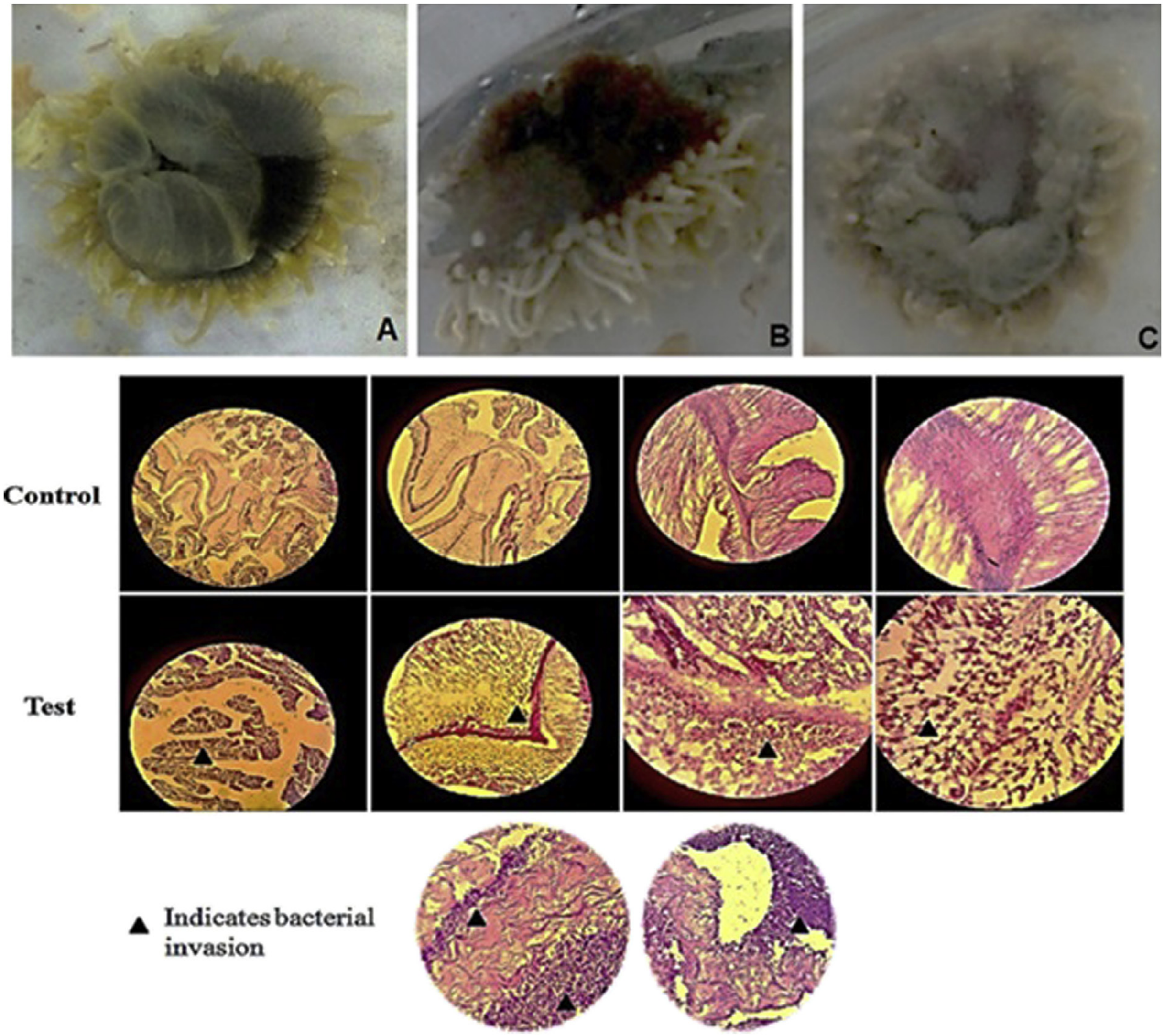

Fig. 7. Results of challenge experiments in *Aiptasia* sp. A. Uninfected sea anemone maintained under sterile condition in sterile seawater found alive throughout the experiment for 48 h. B and C are showing infected sea anemone due to challenge with 10^8 CFU/ml of *S. sciuri*. Infected sea anemone was showing tissue necrosis and mortality between 24 and 48 h. Histopathological examination of infected *Aiptasia* sp. The cell wall and inner protoplasmic content remain intact in control *Aiptasia* sp. whereas test showed fragmented cell wall and damaged inner protoplasmic contents.

confirmed in various assays include auto-aggregation and co-aggregation. On screening of all the isolates for biofilm formation, seven isolates showed positive results for biofilm formation. Biofilm forming isolates produced black coloured colonies with a dry crystalline consistency in congo red agar plates. Based on the intensity of colour developed the isolate D26 was identified as a biofilm producer and cell surface hydrophobicity was observed (Fig. 3A and B). The strain D26 was exposed to various stress factors to determine their influence on biofilm formation. Among the stress factors tested, UV exposure showed the strong influence on biofilm formation in D26 (Fig. 3C). The EPS helps microbes to resist extreme environmental stress in marine environment such as temperature, UV, pollution, agitation, salinity, and depletion of nutrients [43]. This study revealed that increase in UV exposure induced higher biofilm formation as compared to others stresses (agitation, pollution, and salinity). It was found that biofilm formation was induced by environmental stress which can be recorded in the following order UV > pollution > agitation > salinity. Solar ultraviolet radiation is potentially harmful to reef corals. UV radiation can readily penetrate into clear seawater and reef building corals. High sea surface temperature during El Nino southern oscillations is considered as a large-scale environmental stress.

The tube and microtiter plate assays confirmed the production of biofilm (Fig. 3D1-D3). Biofilm was observed as wrinkled colony development (Fig. 4A–C) on different time intervals of 12, 24 and 48 h respectively. Biofilm formed at different time intervals showed variance in colony development. SEM observations proved the biofilm formation capacity of the isolate D26 on a coverslip and is shown in Fig. 4. Biofilm formation was further confirmed by CLSM and the images shown in (4 F-I) are the biofilm formation of D26 on glass wool. There is no evidence of *S. sciuri* as a coral pathogen in the literature. However, *S. sciuri* was known as a multi-host pathogen which causes several diseases in animals, human, and invertebrates [44,45]. *S. sciuri* D26 was identified in this study as an opportunistic pathogen in corals. One of the studies have reported *Serratia marcescens*, a human pathogen isolated from wastewater as a pathogen associated with coral disease acroporid serratiosis [41].

3.4. Cell-cell aggregation in biofilm forming S. sciuri D26

The biofilm forming bacteria *S. sciuri* D26 was examined for both auto-aggregation and co-aggregation. The results showed that *S. sciuri* D26 has the potential to aggregate itself and with other species (D25). It

exhibited auto-aggregation and co-aggregation of upto 70% at 24 h incubation (Fig. 5A). A cellular factor, EPS responsible for aggregation was estimated as 0.014 mg/ml. The FT-IR analysis revealed the presence of cell wall protein with amide groups at (1413 and 1633) and C-H groups (2923),–OH groups (3415) in the extracted EPS (Fig. 5B). The coral-bacteria adhesion experiments revealed that the biofilm forming bacteria invades coral tissue within 24 h. Approximately, 60% of the bacterial cells remain adhered to the coral in 12 h and the number of adhered bacterial cells increased to 80% in 24 h. Un-inoculated control coral fragments showed no adhered bacterial cells in the coral surface and in the internal tissue (Fig. 6A–D).

3.5. Pathogenicity of S. sciuri D26 in model organism sea anemone

The uninfected sea anemone *Aiptasia* polyps were found alive and healthy throughout the experiment under sterile condition. The uninfected polyps were able to survive without any morphological changes (Fig. 7A). The *Aiptasia* polyps infected with a dosage of 10^8 CFU/ml of D26 were found dead after 24 h (Fig. 7B). Immediately after infection, the morphology of the *Aiptasia* polyps changed and after 24 h the polyps showed retraction of tentacles. On observation, after 48 h complete tissue degradation with swollen darkened appearance and mortality was recorded in all infected *Aiptasia* polyps (Fig. 7C). Histopathological analysis revealed that the healthy fragment of tissue was found intact without any damage. Both the cell wall and inner protoplasmic content was intact. But in case of test sample, fragmented cell wall and inner protoplasmic contents were damaged due to bacterial invasion and in some areas, complete loss of protoplasmic content was observed (Fig. 7).

To confirm the pathogenesis *in vivo*, a model organism *Aiptasia* was used in the challenge experiments. The *Aiptasia* has been used in previous reports [46] to study coral pathogenesis. Histopathological analysis revealed that *Aiptasia* sp. was severely infected by *S. sciuri* D26 and it had invaded *Aiptasia* tissue after challenge. The frequency of coral diseases has increased significantly over the last 10 years, causing widespread mortality among reef-building corals. Virulence and pathogenicity of such bacteria are often enhanced when a pathogen forms as a biofilm. New strategies are therefore required to control biofilm formation so as to contain disease causing opportunistic bacteria in corals. Biofilm formation has been found to enhance virulence and pathogenecity in many organisms [47]. The analysis of virulence cascade in *S. sciuri* D26 revealed that this strain was capable of producing protease and superoxide dismutase as reported in *Vibrio shiloi*, a coral pathogen [48]. In addition, *S. sciuri* D26 showed reduction of phenoloxidase and melanin extracted from the coral mucus, which may be playing a major role in coral innate immune system [33]. The sampling site, Palk Bay was largely affected by anthropogenic pollution which might be the cause for the introduction of *S. sciuri* D26 in coral habitats (shallow-water reefs). Anthropogenic pollution and high sea surface temperature during El Nino southern oscillations are considered as stress factors that may have culminated the emergence of *S. sciuri* D26 in corals. However, specific disease condition of corals infected by *S. sciuri* D26 requires continuous field monitoring and further investigation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2018.04.037.

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